High-throughput screening of small molecules for bioactivity and target identification in *Caenorhabditis elegans*

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Published online 22 November 2006; doi:10.1038/nprot.2006.283

This protocol describes a procedure for screening small molecules for bioactivity and a genetic approach to target identification using the nematode *Caenorhabditis elegans* as a model system. Libraries of small molecules are screened in 24-well plates that contain a solid agar substrate. On top of the agar mixture, one small-molecule species is deposited into each well, along with worm food (*E. coli*), and two third-stage or fourth-stage larval worms using a COPAS (Complex Object Parametric Analyzer and Sorter) Biosort. Three to five days later the plates are screened for phenotype. Images of the wells are acquired and archived using a HiDI 2100 automated imaging system (Elegenics). Up to 2,400 chemicals can be screened per week. To identify the predicted protein target of a bioactive molecule, wild-type worms are mutagenized using ethylmethanesulfonate (EMS). Progeny are screened for individuals resistant to the molecules effects. The candidate mutant target that confers resistance is then identified. Target identification might take months.

INTRODUCTION

The use of small bioactive molecules to disrupt protein activity has many advantages over genetic loss-of-function alleles[1]. Small molecules with high target specificity can be used to control the level and timing of protein activity without additional changes to the organism or its environment that are associated with drug-inducible transgenes or temperature-sensitive alleles, respectively. Unlike genetic lesions, bioactive molecules are transferable reagents and may be useful in a wide variety of systems provided that the target is conserved—which is not uncommon[2–5]. Bioactive molecules that disrupt protein targets involved in disease are potential drug leads. Small bioactive molecules with high target specificity are therefore powerful tools for biological analysis.

The simple anatomy and rich genetics of the tiny animal model organism *C. elegans* make it a powerful platform for the discovery and characterization of small bioactive molecules. The cell lineage of *C. elegans* is known and largely invariant[6,7], and its entire genome has been sequenced[8]. It is optically transparent, enabling facile observation of cells with or without the aid of fluorescent reporters of gene expression. High sequence conservation compared with other animals and the ability to model human disease[9] further compound the utility of this nematode worm as an important tool for the discovery of novel drug leads. Finally, a short life cycle of ~3 d (days), small size, diploidy, hermaphroditism, and ease of cultivation make *C. elegans* a unique whole animal model system that is amenable to high-throughput techniques. For these reasons, using *C. elegans* to investigate chemical biology is gaining popularity[2–4,10–12].

To identify new small-molecule tools to probe the biology of *C. elegans*, we developed a protocol to screen libraries of small and structurally diverse molecules for new bioactivities in a high-throughput fashion (**Fig. 1**). The throughput of the screen is greatly enhanced by two key machines. The first is the COPAS Biosort (Union Biometrica), which we use to dispense precise numbers of late third-larval-stage (L3) or early fourth-larval-stage (L4) parental worms into the multiwell plates. This machine rapidly sorts objects based on several criteria including length, optical density, and two channels of fluorescence. Empirically determined selection criteria of length and optical density were used to deposit two late L3 or early L4 worms into each well of the multi-well plate in an automated fashion. The COPAS Biosort minimizes the manual manipulation of individual worms[13] and therefore dramatically increases throughput of the screen.

The second machine that facilitates throughput of the small-molecule screen is the high-throughput digital imager, or HiDI 2100 (Elegenics) (**Fig. 1**). In the planning stages of the screen, no commercially available device could image and analyze samples residing on top of semiopaque substrate in multiwell plate format in a high-throughput manner (see ref. 14 for a review of current imaging devices). We therefore constructed a versatile imaging device that would enable image acquisition from above. It is divided into two functionally distinct systems: a plate management system and an image acquisition and management system. The two systems communicate with each other to coordinate plate delivery and retrieval, to accept commands, and to provide status. HiDI is designed to run unattended, but can be monitored and controlled via the internet. The plate management system makes use of a robotic arm (Mitsubishi, RV-2AJ-S11) as the plate mover. The system accommodates a variety of single and multiwell plate formats and can process up to 104 plates autonomously. Barcodes on the plates and a reader on the arm track the plates and dictate the analysis executed by HiDI.

The image acquisition and management system of HiDI was designed to acquire images of an entire well of a 24-well plate (1.7 cm in diameter), while having the capability to resolve

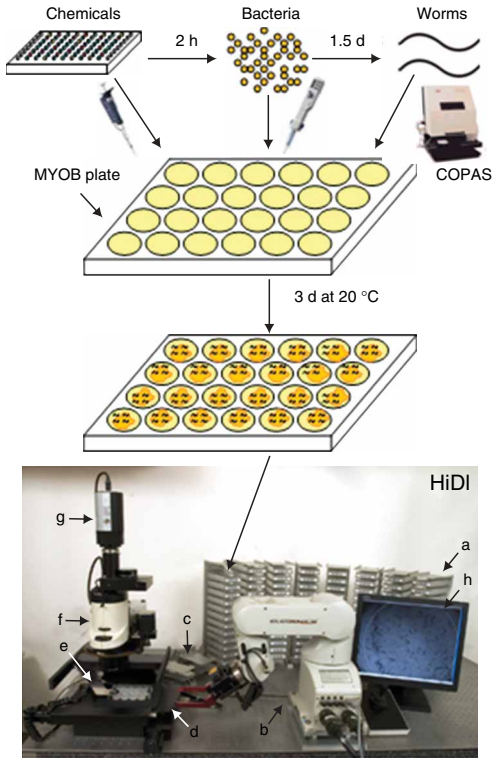

Figure 1 | High-throughput small-molecule screen. First, chemicals are added to the 24-well MYOB plates. Bacteria are then added to each of the wells. After the bacteria have dried, two L3 or L4 worms are deposited in each well. The worms are incubated at 20 °C for 3 d. After 3, 4 and 5 d the plates are screened for chemical-induced phenotypes. Images of every well are acquired in an automated fashion using the HiDI 2100 high-throughput digital imager. Components of the HiDI include: stacked plate trays (a); robotic arm (b); plate lid removal and replacement station (c); motorized stage (d); CO_2-delivery arm (e); modified Leica MZ16A dissection microscope (f); cooled charge-coupled device (CCD) camera (g); and image output and data storage (h).

individual axons expressing fluorescent reporters. The limits of optical magnification of the system are therefore ×0.45 to ×23 and were achieved by mounting a high-speed objective turret harboring ×0.63 and ×2.0 apochromatic objectives on a modified Leica MZ16A motorized stereomicroscope. We chose the MZ16A to accommodate the long working distances required to focus into the microplate wells. At high magnification or in epifluorescent mode, animals are reversibly anesthetised with CO_2, which is delivered via a custom mechanical arm (**Fig. 1**) to enable longer image-acquisition times. Images are captured with an Evolution QEi cooled monochrome 12-bit CCD camera with 1392 × 1040 pixel resolution (Mediacybernetics). Image Management Software is used to set-up the analysis protocols and to control the imaging system during a run. Once barcoded plates are loaded onto the HiDI storage trays, the system acquires the desired images in an automated fashion.

The images of the phenotypic effects induced by nearly all molecules from our published screen were acquired and archived using HiDI[2]. Examples of some typical images acquired by HiDI are shown in **Figure 2**. We investigated the correspondence between scoring phenotype from the archived images and independently scoring phenotype manually at the dissection microscope for 1889

Screen the plates 3, 4 and 5 d after seeding worms

of these compounds. We were able to detect 72% of the phenotype by both methods, 17% by HiDI alone and 11% by manual inspection alone. We therefore conclude that scoring phenotype from HiDI-acquired images is as good as or better than manually scoring phenotype at the dissection microscope, although the former enables the detection of subtle effects on population growth, and the latter enables the detection of more subtle defects in movement that cannot be assayed from a single still image.

To identify the predicted protein target of a bioactive molecule, we performed a forward genetic screen designed to enrich for mutations within the gene encoding the target protein of nemadipine-A (**Fig. 3**)[2]. There are several reviews of the genetic screens previously done to recover *C. elegans* mutants resistant to a bioactive molecule[9,12,15,16]. We assumed that the molecule was an antagonist and reasoned that there may be amino acid residues within the target whose substitution will disrupt interaction with the small molecule without disrupting biological function. These neomorphic mutations will be dominant over the wild-type gene product in the presence of the antagonist and will be revealed in the

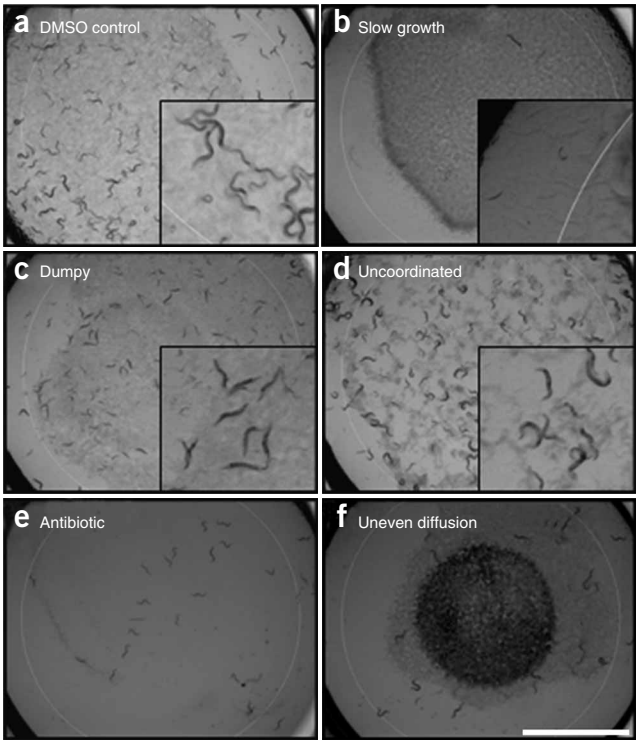

Figure 2 | Typical images generated by HiDI. All images were acquired with bright-field illumination 4 d after two L3 hermaphrodites were deposited in the wells. Each phenotype described is chemically induced. (**a**) Worms grown on 0.5% DMSO have normal shape, proportions and brood size. (**b**) Slow-growing worms take longer to populate the well. (**c**) Dumpy worms have a shortened stature compared with the wild type. (**d**) Uncoordinated worms do not show typical sinusoidal movement, which is further evidenced by uneven food distribution. (**e**) The chemical in this well is an antibiotic; no bacterial lawn is visible despite low brood count. (**f**) This chemical does not diffuse through the agar, and remains concentrated at the center of the well. Insets in **a**–**d** show ×3 magnification of selected areas in the well. Scale bar, 5 mm.

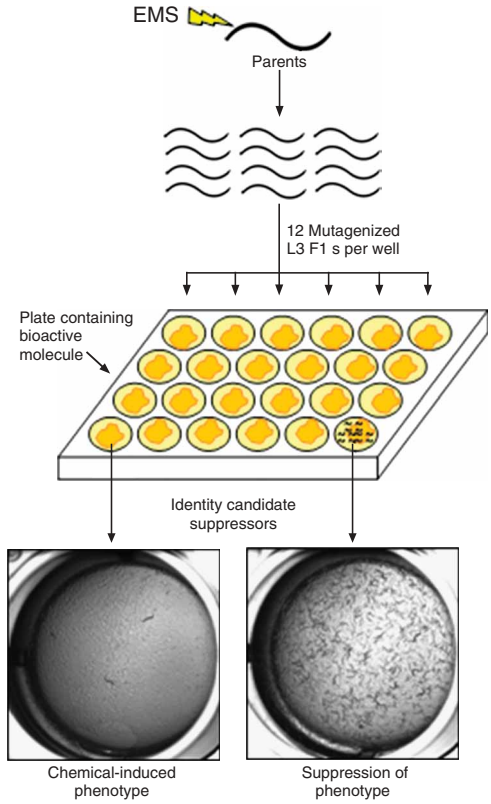

Figure 3 | The F_1 suppressor screen. A mixed population of worms enriched for L4 stage worms are mutagenized with EMS. Twelve mutagenized L3 F_1 worms are then deposited in each well of the 24-well chemical plates. The plates are screened on a day when the drug-induced phenotype usually appears in similarly staged wild-type worms. Candidates are identified by their suppression of the phenotype.

first filial (F_1) generation produced by mutagenized parents. An F_1 screen might also reveal hypermorphic mutations that confer resistance to an antagonist, because increased target activity may decrease the effective concentration of the molecule in hypermorphic individuals in a dominant fashion. In only the most unusual of circumstances will loss-of-function mutations yield the targeted protein of an antagonist[17]. Although we have provided proof-of-principle that an F_1 screening strategy efficiently reveals the target of an antagonist[2], screens for mutants resistant to the acetylcholine esterase inhibitor aldicarb have been unsuccessful in identifying the target genetically for unknown reasons[12,18,19].

Genetic screens for *C. elegans* mutants resistant to agonists can also identify the predicted protein target, as demonstrated for the agonists levamisole[20,21] and ivermectin[22,23]. For each of these compounds, F_2 screens were done to enrich for loss-of-function mutations that confer resistance, and many positive regulators of the pathway, including the respective targets, were identified. Although this genetic approach provides insight into the genetic pathways of the targeted protein[12], it confounds target identification because many distinct candidates are revealed.

The ease of isolating mutants resistant to an antagonistic molecule in an F_1 screen will depend on several factors: First, the antagonist may have more than one physiologically relevant target, each of which leads to phenotype when disrupted. The frequency of isolating resistant mutants is therefore the product of the frequency of isolating each mutation alone, making double-mutant isolation at least thousands of times less frequent than if there were a single target. Second, only an extremely rare mutagenic event may disrupt the interaction between the bioactive molecule and the protein target without disrupting biological function. It may also be a very rare mutagenic event that increases the activity of the gene product to make the individual effectively less sensitive to the compound. However, these concerns are mitigated in part by the fact that hundreds of thousands of *C. elegans* genomes can be screened in a relatively short period depending of the nature of the screen. Alternatively, it may not be possible to mutate the protein target to a hypermorphic or relevant neomorphic state. Third, although rare in *C. elegans*, the gene product may be haploinsufficient. Thus, even if one of the two copies of the gene product is mutated to insensitivity, the F_1 mutant individual remains sensitive to the antagonist because the animal cannot tolerate fewer than two gene doses. Fourth, the phenotypic penetrance, or the percentage of individuals within a population that exhibit the phenotype, will dramatically impact any screen for mutants resistant to a bioactive molecule. A phenotypic penetrance of less than 100% will inundate a screen with false positives, each of which will have to be retested and will greatly retard progress. Finally, the nature of the phenotype will also dramatically impact the progress of the screen. Having to mount samples on a slide to observe phenotype will require Herculean efforts to recover resistant mutants compared with phenotypes readily observed on a Petri dish with a dissection microscope. However, one advantage of working with bioactive molecules is that a phenotypic series is typically generated upon increasing concentrations. A molecule that induces only subtle effects at lower concentrations can have lethal consequences at higher concentrations. For example, a nemadipine-A concentration as low as 0.5 μM induces egg-laying defects, but a concentration 25 times greater is required to retard population growth[2]. Thus, resistant mutants can be selected for at higher concentrations of the bioactive molecule, and then further investigated to determine whether all compound-induced phenotypes are mitigated by the same mutation. Thus, there are many important factors to consider before initiating a genetic screen to identify the target of a bioactive molecule.

There are several considerations to the methods described here. First, all small-molecule solutions are described as being handled manually. Manual addition of the small molecules to the plates is the most labor-intensive component of the preparative phase and would ideally be automated. There are several commercially available liquid-handling robots that could be adapted to serve this purpose. Second, it is not possible to determine whether a novel bioactive compound is an agonist or antagonist from phenotypic analysis alone, which could impact the approach taken for target identification. Third, it will likely be unclear if an F_1 or an F_2 screen will be the best strategy for target identification. As discussed above, F_1 screens could reveal the target of an antagonist through the generation of dominant neomorphic or hypermorphic mutations within the target[2]. Although lacking experimental proof in *C. elegans*, F_1 screens could also reveal the target of an agonist. Heterozygous hypomorphic mutations within the target could make F_1 individuals resistant to a certain concentration range of

the agonist because of reductions in the overall activity of the gene product. The targets of agonists and antagonists alike should be revealed in F_2 screens as they generate both dominant and recessive mutations[17,20–23]. Thus, the safe genetic approach towards target identification is an F_2 screen, although it can represent a significant increase in labour because protocols need to be in place to ensure the resulting mutations are independent and not derived from the same mutant F_1 worm. Fourth, although thousands of small molecules can be screened each week, it takes at least several months to genetically define and characterize candidate targets of any one compound, therefore making prioritization of the bioactive 'hits' from a small-molecule screen critical.

The methods described here are not limited to the use of wild-type *C. elegans*. For example, small-molecule screens could be done to correct pathological states[9] generated by mutations, siRNAs, transgenes or even other small molecules. The same libraries of small molecules could be screened against species related to *C. elegans*, such as *Caenorhabditis briggsae* or *Caenorhabditis remanei*, or other types of nematodes to investigate pharmacogenomic variation. Small molecules that disrupt cellular or subcellular processes could also be screened for by screening worm strains that express fluorescent reporters. Hopefully, many more small-molecule screens will be performed in the future to generate new tools that will complement the powerful genetics of *C. elegans*.

MATERIALS

REAGENTS

- Trizma HCl (Sigma, cat. no. T-5941)
- Trizma base (Sigma, cat. no. T-1503)
- Bacto tryptone (BD Difco™)
- Cholesterol (95%) (Sigma, cat. no. C8503)
- Agar (Sigma; cat. no. A-1296)
- OP50 *Escherichia coli* (*C. elegans* Genetic Center, www.cbs.umn.edu/CGC/)
- Bio-tryptone (BioShop Canada, cat. no. TRP402)
- Yeast extract (BD Difco™)
- Small-molecule libraries (e.g., Sigma Inc, Microsource Discovery Inc., Chembridge Inc., Maybridge Inc.) **! CAUTION** The small-molecule libraries may contain toxic compounds.
- Dimethylsulfoxide (DMSO) (EMD Chemicals, cat. no. MX1458-3)
- Ethylmethanesulfonate (EMS) (Sigma, cat. no. M0880) **! CAUTION** EMS is a mutagen.
- 10% NaOCl bleach solution (Sigma, cat. no. 425044)
- NaOH (Caledon Laboratories, cat. no. 7860-1)
- Na_2HPO_4 (EMD Chemicals, cat. no. SX0720-1)
- KH_2PO_4 (EMD Chemicals, cat. no. P0662)
- $MgSO_4 \cdot 7H_2O$ (BioShop Canada, cat. no. MAG511)
- SURVEYOR™ Check-It Kit (Transgenomic, cat. no. 706040)

EQUIPMENT

- 24-well tissue culture plates (Greiner bio-one, cat. no. 662160)
- 60 × 15 mm culture plates (Fisher Scientific, cat. no. 08-757-13A)
- COPAS Biosort (Union Biometrica)
- MZ12 dissection microscope (Leica)
- HiDI 2100 high-throughput digital imager (Elegenics)
- Nutating Mixer (VWR International, cat. no. 82007-202)

REAGENT SETUP

MYOB dry mix (for 370 g) 27.5 g Trizma HCl, 12 g Trizma base, 230 g bacto tryptone, 100 g NaCl, 0.4 g cholesterol (95%), mix with shaking.

MYOB medium (for 1 liter) 7.4 g MYOB dry mix, 22 g agar, make up to 1 liter with ddH_2O, autoclave.

LB growth medium (for 1 liter) 10 g bio-tryptone, 5 g yeast extract, 10 g NaCl, make up to 1 liter with ddH_2O, adjust pH to 7.0, autoclave.

1 M NaOH (for 500 ml) 20 g NaOH, make up to 500 ml with ddH_2O.

M9 buffer (for 1 liter) 6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, make up to 1 liter with ddH_2O, autoclave, let buffer cool to room temperature (RT; 22 °C) and add 2 ml of 1 M $MgSO_4 \cdot 7H_2O$.

Alkaline bleach solution (for 9 ml) 2 ml 10% NaOCl bleach solution, 5 ml 1M NaOH, 2 ml ddH_2O.

PROCEDURE

High-throughput small-molecule screen

1| Prepare 1 liter of MYOB medium in a 2-liter flask.

2| After autoclaving, allow MYOB medium to cool for 1 h in a 60 °C water bath.

3| Using a repeater pipette, add 1 ml warm MYOB medium to each well of forty 24-well tissue culture plates.
▲ CRITICAL STEP It is our experience that this protocol performed in our environs results in little variability in the dryness of the multiwell plates. However, people from different environs report that drying of the multiwell plates is variable. We therefore recommend careful monitoring of the plates as they dry to minimize plate-to-plate and well-to-well variability in the dryness of the plates. See Step 10 and TROUBLESHOOTING for more information.

4| Using sterile technique, inoculate a 100-ml culture of LB growth medium from a single colony of OP50 *E. coli*.

5| Let 24-well plates and OP50 culture stand overnight at RT.

6| Refrigerate overnight OP50 culture at 4 °C (OP50 are at stationary phase).

7| Use a pipette to add small molecules from the libraries to each well of the 24-well plates (do this work in a sterile laminar flow hood). A final small-molecule concentration of 25 μM and a final solvent (usually DMSO) concentration of $<0.5\%$ is desired (assuming complete diffusion in the agar). Keep the final concentration of DMSO below 0.5%. High concentrations of DMSO can result in developmental defects or lethality. Add the small molecules to the surface of the agar. There should be one small molecule per well. Each small molecule should be assayed in duplicate, with replicates in adjacent wells. Forty 24-well plates are adequate for screening 480 small molecules in duplicate. Let the 24-well plates stand at RT for 2 h.
! CAUTION The small-molecule libraries may contain toxic compounds.

▲ CRITICAL STEP There must only be one small molecule per well. We found that pooling eight small molecules per well yields tenfold fewer phenotypes ($n = 10{,}000$) (R. Fraser, P.M. and P.J.R., unpublished results). The reason for this is unclear. Assaying each small molecule in duplicate is important so that phenotypes can be verified immediately.
? TROUBLESHOOTING

8| Use a repeater pipette to add 25 µl OP50 *E. coli* culture, prepared in Step 4, to each well of the 24-well plates. Sterile technique should be used.

9| Let plates stand overnight at RT.

10| Dry plates for ~1 h in a sterile laminar flow hood (or until bacterial lawn is dry).
▲ CRITICAL STEP The bacterial lawns in all of the wells must be dry, otherwise there may be inconsistent worm growth from well to well. The plates should not be over-dried, as this could cause cracks to form in the agar.
? TROUBLESHOOTING

11| Let plates stand overnight at RT.

12| Use the COPAS Biosort to deposit two late L3 or early L4 hermaphrodite worms per well of the plates. The worms should be taken from a mixed-stage population. Culture worms according to established protocols[13].
▲ CRITICAL STEP Only two wild-type late L3 or early L4 parents are deposited per well because this number and stage consistently maximizes the number of individuals in the F_1 generation that reach adulthood without depleting the food in DMSO control wells. The inclusion of more than two parents per well of a 24-well plate containing the OP50 strain of *E. coli* results in an overabundance of F_1 worms that starve before reaching adulthood, making phenotypic assessments unreliable. Rarely, a parent dies after COPAS deposition, thereby necessitating two worms per well to ensure a robust assay. If a COPAS Biosort is not available, it is recommended to manually place two late L3 or early L4 parents per well for small screens. For larger screens, ~100 L1 hatchlings synchronized from an embryo preparation (see Steps 27–32) could be deposited into each well using a repeater pipette.

13| Store the plates at 20 °C for 3 d.

14| Manually screen each well of each plate with a dissection microscope 3, 4 and 5 d after depositing the late L3 or early L4 worms on the plates. Observe and note any interesting phenotypes. Acquire and save images of the wells using the HiDI 2100 automated high-throughput digital imager. Take full-well and magnified close-up pictures of each well. Screen through the images generated by HiDI, and note any interesting phenotypes.
■ PAUSE POINT Steps 1–14 constitute one cycle of screening. The procedure can be stopped after every cycle, although it is desirable to finish screening all available small molecules before pausing.

Target identification of small molecules

15| Prepare MYOB medium as in Steps 1–2.

16| Add the bioactive small molecule of interest to the media after it has cooled to 60 °C. The final concentration of the bioactive small molecule should be about 1.5 times higher than the effective concentration at which 50% of the individual worms exhibit the phenotype (EC_{50}). The EC_{50} can be determined from a dose-response curve for the small molecule.

17| Using a repeater pipette, add 1 ml warm MYOB medium + bioactive small molecule to each well of an appropriate number of 24-well tissue culture plates.

18| Let plates dry overnight at RT.

19| Add OP50 *E. coli* to the plates and dry as in Steps 8–11. These plates will be used in Steps 35–40.
■ PAUSE POINT Depending on the stability of the bioactive small molecules, the plates can be stored for up to 30 d at 4 °C.

EMS mutagenesis

20| Perform EMS mutagenesis[24] on a population of wild-type worms enriched for the L4 larval stage. Collect healthy wild-type worms from six densely populated 6-cm MYOB plates by washing with M9 buffer and depositing them into a 15-ml conical tube.

21| Wash the worms three times with M9 buffer. One wash is performed by filling up the 15-ml conical tube with M9, pelleting the worms for 2 min at 796*g* (2,000 r.p.m. in a benchtop Eppendorf 5810 R centrifuge) and aspirating the supernatant. This is to minimize the amount of OP50 *E. coli* remaining in solution with the worms. In the final wash, aspirate to a final volume of 5 ml.

22| Add 25 µl EMS (final concentration 50 mM) to 5 ml of worms. Mix well with gentle inversions of the tube as EMS settles to the bottom and may stick to the plastic.

! CAUTION EMS is a mutagen. EMS causes nucleotide mutations in DNA, and is probably carcinogenic. Perform Steps 22–25 in a fume hood, wear protective clothing (lab coat, lab goggles and double gloves) and ensure overall careful handling is observed. Also, be sure to keep washes in an EMS-specific waste container and discard EMS waste according to institutional policies. EMS can be inactivated or detoxified with 1 M NaOH[24,25].

23| Incubate the worms in EMS for 4 h at RT on a nutator or with manual inversions every 0.5 h.
▲ CRITICAL STEP Take care when mutagenizing worms. If the worms are not mutagenized enough, more genomes than necessary will have to be screened before finding a mutant of interest. If the worms are too heavily mutagenized, the mutants of interest may carry a higher proportion of unwanted background mutations.

24| At the end of the mutagenesis, wash the worms five times as previously described (see Step 21). These washes are necessary to minimize EMS in solution.

25| Deposit all mutagenized worms onto 6-cm MYOB plates with OP50 *E. coli*. Use enough plates so that the worms will not starve before 24 h of growth.
▲ CRITICAL STEP If the worms starve before 24 h, the yield of mutagenized F_1 worms will be low.

26| Store the plates for 24 h at 20 °C.

Embryo harvest

27| Use a bleach digestion to harvest embryos from the mutagenized parental worms and, in parallel, from non-mutagenized wild-type adults (the non-mutagenized worms will be used as negative controls in Steps 35–37). Suspend washed worms in 1.5 ml M9 buffer in a 15-ml conical tube. Ideally the washed worms should be enriched for adults that contain eggs. There should be no more than 500 µl of packed worms in the 1.5-ml suspension.

28| Add 4.5 ml alkaline bleach solution to the worm suspension. Invert the tube immediately, two times. Invert the tube once every 30 s for 4.5 min to prevent the worms from collecting at the bottom of the tube.
▲ CRITICAL STEP The alkaline bleach solution should be made fresh every time. The 10% NaOCl bleach solution should be stored at 4 °C.

29| Vortex the tube for 10 s. Add 6 ml M9 buffer to the digest, and invert the tube two or three times. Immediately centrifuge the tube for 2 min at 796*g* (2,000 r.p.m. in a benchtop Eppendorf 5810 R centrifuge).
▲ CRITICAL STEP It is important to get the embryos out of the alkaline bleach solution as quickly as possible after the 4.5 min of digestion. In general, digestions longer than 5 min will result in a higher proportion of dead embryos. However, digestions that are stopped prematurely (i.e., <4 min) will result in less-efficient digestion.

30| Aspirate the supernatant. Resuspend the worms in 10 ml M9 buffer. Shake the tube vigorously until the pellet is resuspended. Immediately centrifuge the tube for 2 min at 796*g*.
▲ CRITICAL STEP Without vigorous shaking of the tube the embryos will remain in a clump at the bottom of the tube, and will not be adequately washed. Insufficient washing will result in decreased viability of the embryos.

31| Repeat Step 30 four more times. After the fourth aspiration, resuspend the embryos in 5–10 ml M9 buffer.
? TROUBLESHOOTING

32| Incubate the F_1 embryos on a nutating mixer overnight at 20 °C.

33| Deposit all of the resulting L1 hatchling worms onto 6-cm MYOB plates with OP50 *E. coli*. Use enough plates so that the worms will not starve after 2 d of growth at 20 °C. Be sure to keep the mutagenized and non-mutagenized worms separate. Alternatively, L1 hatchlings may be deposited directly into the 24-well plates by proceeding directly to Step 35. Choosing to deposit L1 hatchlings or L3 worms into the multiwell plates will depend on which life-stage the bioactive molecule affects. If the molecule affects all stages relatively equally, then we recommend depositing L3 worms because it is more likely that these animals will reach adulthood under control conditions and therefore yield a more consistent assay. We do not recommend depositing embryos because many die from the bleaching procedure (Steps 27–31), thereby yielding a variable number of animals that hatch.
▲ CRITICAL STEP It is important that worms other than hatchlings do not starve. Starvation causes unwanted physiological stress for the worms. Therefore, starved worms should not be deposited on the plates for screening in Steps 35–40.

34| Store the plates for 48 h at 20 °C.

35| Use the COPAS Biosort to deposit twelve L3 mutant F_1 worms per well of the 24-well plates containing the bioactive molecule, made in Steps 15–19. Also deposit twelve L3 non-mutagenized F_1 worms per well of the 24-well plates containing the

bioactive molecule as a negative control. From Steps 27–34, there will be enough F_1 mutant worms to place into fifty 24-well plates and typically a vast surplus.
▲ CRITICAL STEP Make sure that all of the wells contain a similar number of worms (±2). Differences in the number of worms deposited may confound screening.

36| Incubate the plates at 20 °C.

37| Pick a day to screen through the plates when the phenotype induced by the bioactive small molecule is evident in the non-mutagenized wild-type control plates. Identify wells containing worms that are resistant to the effects of the compound.
▲ CRITICAL STEP Assume only one F_1 worm in each of the candidate wells gave rise to the resistant individuals. The resistant worms in one well are genetically distinct from those of another well, and care must be taken to keep all lines separate from each other. Thus, naming each line of resistant worms derived from distinct wells is essential.

38| To determine whether the resistant phenotype breeds true, clone twelve L3 F_2 worms isolated from a well containing mutants of interest into separate wells of the 24-well plates made in Steps 15–19, taking care to keep track of which well each individual came from. For consistency, the concentration of the bioactive molecule in the plates should be the same as the concentration that was used for the F_1 screen. Wild-type worms should also be cloned into the 24-well plates as a negative control.

39| Incubate the plates at 20 °C.

40| Screen the progeny of the F_2 clones for resistance to the effects of the molecule on a day when the phenotype is obvious in the negative control plate. If many of the wells contain resistant F_3 worms, then the resistant phenotype breeds true. Identify about six wells that have the most resistant animals, as these will have the best chance of being homozygous. Test for homozygosity by cloning twelve L3 F_3 worms from each of the six resistant wells into separate wells of the 24-well plates made in Steps 15–19, again taking care to keep track of which well each individual came from. If all 12 F_3 worms derived from a single F_2 give rise to resistant F_4 worms, then a homozygous line has probably been isolated. If not, repeat Step 40.
▲ CRITICAL STEP Take care to keep track of independent lines derived from distinct F_1 worms.
■ PAUSE POINT Resistant mutant lines should be frozen at this point[25]. Mapping of the mutations can resume at any time by growing the worms from a thawed sample or from an old starved plate.
? TROUBLESHOOTING

● TIMING

High-throughput small-molecule screen
Steps 1–4, 2 h
Step 5, overnight
Step 6, overnight
Steps 7–8, 7 h
Step 9, overnight
Step 10, ~1 h
Step 11, overnight
Step 12, 3 h
Step 13, 3 d
Step 14, 9 h
Steps 1–14 constitute one cycle of screening. Two screening cycles can be started each week. In this manner 960 small molecules can be screened in duplicate each week. It should be noted that with one HiDI system, up to 1,200 chemicals could be screened in duplicate each week.

Identification of small-molecule target genes
Steps 15–17, 3 h
Steps 18–19, 2 d
Steps 20–25, 6 h
Step 26, 24 h
Steps 27–31, 1 h
Step 32, overnight
Step 33, 20 min
Step 34, 2 d
Step 35, 3 h

Step 36, 3+ d
Step 37, 2 h
Step 38, 30 min
Step 39, 3+ d
Step 40, 2 h

? TROUBLESHOOTING

See **Table 1** for troubleshooting advice.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
7	The small molecule precipitates in the solvent.	The small molecule has been frozen and thawed too many times. DMSO is very hygroscopic and becomes hydrated on repeated exposure to air; this can cause compounds that were once soluble in 100% DMSO to crash out of solution.	Thaw the library plates only when absolutely necessary.
		The solution is saturated.	If there is visible precipitate, be sure to resuspend as much of it as possible before transferring the small molecule. After the screen is complete, individual dose curves can be done for bioactive small molecules using fresh stock solutions.
	The small molecule does not diffuse evenly throughout the agar.	The small molecule is insoluble in water or does not diffuse readily through the agar substrate.	After the screen is complete, a dose-response curve can be made for the small molecule. The plates used for dose-response curves are made by physically mixing the small molecule in the media (as in Steps 16–18), rather than relying on diffusion.
10	There are cracks in the agar.	The plates were over-dried.	Reduce drying time in the flow hood by 5-min intervals until a time is reached when the bacteria are dry but the wells are not cracked.
	There are pools of liquid in the bacterial lawns.	The plates were not dried adequately.	Increase drying time in the flow hood by 5-min intervals until a time is reached when the bacteria in all of the wells are dry.
	The wells in the centre of the plate will not dry.	The plates were not dried adequately or were dried with the lids on.	It is best to use a sterile flow hood when drying the plates. The lids of the plates should be removed before drying otherwise the innermost wells of the plates may not dry. If there is a large difference in the dryness of the inner versus outer wells, place an old sterile lid on the plate with the center cut out of it with a red-hot scalpel.
31	There is a low yield of viable L1 hatchlings after the embryo preparation.	The worms were overbleached.	Shorten the bleaching step of the embryo preparation by 15-s intervals until the L1s are viable.
		There were not enough adults in the starting population.	Make sure the starting population of worms is enriched for adults before performing the embryo preparation.
40	The suppressors cannot be homozygosed.	The mutation may be homozygous lethal or it may be linked to a mutation which is homozygous lethal.	The mutation can be maintained by making it in trans to a genetic balancer[27].

ANTICIPATED RESULTS

High-throughput small-molecule screen

Roughly 2–3% of the small molecules screened from a given library might induce obvious defects in wild-type worms[2,20]. However, this 'hit rate' will depend on the nature of the small-molecule library. For example, 1.7% of small molecules with unknown bioactivity induced gross phenotypes in the worm ($n = 10{,}000$) yet 3.2% of the molecules screened from libraries

enriched for molecules with bioactivity in other systems induced phenotype ($n = 3,280$). The hit rate will also greatly depend on the range of phenotypes considered and the ability to detect them.

Identifying the targets of small molecules

Once mutants resistant to the bioactive molecule have been isolated, several methods exist for mapping and identifying the mutated gene. Molecular[26] and/or visible[20] markers can be used to quickly identify a small chromosomal region within 500 kb of where the mutation resides. Attractive candidates can then be screened for polymorphisms using the SURVEYOR™ Check-It Kit[2] (T.C.Y.K. & P.J.R., unpublished results), followed by sequencing of the gene region containing the polymorphism.

Relatively few screens for small-molecule-resistant mutants have been reported to date for *C. elegans*[2,17–23]. In addition, it is likely that different targets will have different capabilities of mutating to resistance. It is therefore difficult to estimate how many mutagenized genomes must be screened to find a candidate target of a bioactive molecule, especially where only neomorphic or hypermorphic mutations will confer resistance. In our screen for mutants resistant to the antagonist nemadipine-A, we found 15 strong candidates out of 180,000 haploid mutagenized genomes, or about one out of every 6,000 F_1 worms was resistant[2] (T.C.Y.K. and P.J.R., unpublished results). Of these mutations, 14 are in *egl-19*, which encodes the target of nemadipine-A, and none are loss-of-function mutations based on preliminary phenotypic characterization. It is currently unclear whether *egl-19* is unusually mutagenic or if the frequency of mutant recovery from this screen will be representative. Of note, a screen for mutants resistant to a second molecule was also useful, resulting in one resistant mutant for every 2,000 F_1 worms (S. Alfred, S.R.C. and P.J.R., unpublished results). As previously discussed, the target of agonists will probably be revealed by hypomorphic, or reduction or loss-of-function mutations[20–23]. The frequency of recovering loss-of-functions in the average-sized gene with the concentration of EMS used here is about one in 2,000 haploid mutagenized genomes screened[20,24], making the recovery of mutants resistant to an agonist straightforward. If one or more mutants resistant to a bioactive molecule are not recovered after screening more than 50,000 genomes, a re-evaluation of the screening approach is warranted.

ACKNOWLEDGMENTS We thank Jonathan Hodgkin suggesting the use of CO_2 to anesthetise worms. We thank Simon Alfred and Regina Fraser for sharing unpublished results, and the anonymous reviewers for insightful suggestions. P.M., S.R.C. and P.J.R. are Canadian Research Chairs in plant molecular biology, plant genomics and molecular neurobiology, respectively. This work was supported by an NSERC Industrial Grant to P.M., and a CIHR Grant, Premier's Research Excellence Award and awards from the Canadian Foundation for Innovation and Ontario Innovation Trust to P.J.R.

COMPETING INTERESTS STATEMENT The authors declare competing financial interests (see the html version of this article for details).

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